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DIFFERENTIATION BETWEEN TYPHOID VI  
AND W ANTIGENS BY 2, 3, 5-TRIPHENYL  
TETRAZOLIUM CHLORIDE INCORPORATED  
IN A MEDIUM

Thesis for the Degree of M. S.  
MICHIGAN STATE COLLEGE  
Grace Maghakian Allen  
1951

This is to certify that the

thesis entitled

Differentiation Between Typhoid Vi and W  
Antigens by 2, 3, 5 Triphenyltetrazolium  
Chloride Incorporated in a Medium.

presented by

Grace Allen

has been accepted towards fulfillment  
of the requirements for

M. S. degree in Bacteriology

Walter H. Mack

Major professor

Date May 16, 1951

DIFFERENTIATION BETWEEN TYPHOID VI AND W ANTIGENS  
BY 2,3,5-TRIPHENYL TETRAZOLIUM CHLORIDE  
INCORPORATED IN A MEDIUM

By

Grace Maghakian Allen

A THESIS

Submitted to the School of Graduate Studies of Michigan  
State College of Agriculture and Applied Science  
in partial fulfillment of the requirements  
for the degree of

MASTER OF SCIENCE

Department of Bacteriology and Public Health

1951

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6/28/51  
gift

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## ACKNOWLEDGEMENT

The author wishes to express sincere appreciation to Dr. H. J. Stafseth, Professor and Head of the Department of Bacteriology and Public Health for his corrections of this manuscript and to Dr. W. N. Mack, Associate Professor of Bacteriology and Public Health, for his advice and counsel.

The author also wishes to express sincere gratitude to Dr. I. F. Huddleson, Professor of Bacteriology and Public Health for his patient instruction of procedures used in this study and to Dr. W. N. Ferguson, Associate Director of the Salmonella Typing Laboratory, Michigan Department of Health, for his helpful suggestions and for supplying the required cultures.

The author also wishes to thank Mr. Roland C. Fulde, Graduate Assistant in the Department of Bacteriology and Public Health, Michigan State College, for the preparation of photographs used herein.



## INTRODUCTION

The reduction in morbidity due to typhoid fever in various parts of the world has been one of the most striking phenomena in modern public health experience. Despite our modern methods of sanitation and public health programs, typhoid fever continues its manifestation as isolated epidemics in rural communities and institutions. In view of this fact, any information concerning the etiologic agent, Salmonella typhosa, is received as a possible basis for better control of the disease.

The discovery of Vi antigen and its association with the virulence of S. typhosa, (Felix and Pitt, 1934) instituted a new field of speculation and research on that organism. The practical results of these investigations are generally evident in our procedures for laboratory diagnoses, specific therapy and prophylaxis. Of these, the introduction of routine bacteriophage typing (Craigie and Yen, 1938) has served as an invaluable aid to the epidemiologist. This method permits the identification of Vi antigen in a culture. It also permits the identification of the specific type responsible for producing the disease in a case, carrier or epidemic.

Basically, the technique for bacteriophage typing requires introducing the effects of one living agent upon another. The simplest alternative for detecting Vi antigen, would be direct observation of characteristic colonial



differences between strains of typhoid bacilli containing Vi and W antigens. Several authors have reported these differences. However, their distinction is necessarily reserved for those who are highly experienced and therefore capable of accurate judgement.

It is desirable to place at our disposal a relatively tractable method for recognizing the presence of Vi or W antigens in a culture. The utilization of 2,3,5-triphenyl tetrazolium chloride has recently been adopted (Levine 1949, Huddleson 1950) for differentiating bacterial variation by colonial observation. In this study, this salt was applied to S. typhosa with the same purpose.



## HISTORY

One of the earliest reports describing the visible effects of variation in typhoid organisms was contributed by Savage (1901). He observed clumping of S. typhosa in old broth cultures simulating agglutination, in contrast to the bacilli which remained dispersed and in active movement in young broth cultures. This was reported due to frequent subcultivation rather than prolonged incubation. He attributed the pseudo-clumping as having some bearing on the susceptibility of the race of S. typhosa.

Essentially the same phenomenon was later detected by Arkwright (1922), Andrewes (1922), White (1929) and Grinnell (1930). Their observations ultimately raised the question regarding the comparative efficacy of vaccines prepared from rough and smooth colonies. Grinnell suggested that some strains utilized for typhoid vaccines may have originated from the rough, less potent variety and others from the smooth, virulent types.

Such evidence was conducive to more thorough investigation by Felix and Pitt (1934a), who reported that roughness in colonies indicated low virulence, while smoothness was an indication of high virulence. However, the mere presence of smooth "O" or somatic antigen did not necessarily indicate high virulence. They determined virulent strains by agglutination tests. Those strains of S. typhosa which demonstrated inability to agglutinate with

smooth "O" antibodies, were termed O-inagglutinable, and proved to be highly virulent. O-agglutinable strains were not as virulent. Since the strains were not distinguishable by smooth or rough origin, it was indicated that some unknown factor was essential for the resistance to O antigen antibody. In that same year, Felix and Pitt (1934) were able to isolate a new antibody independent of the O and H antibodies. The symbol "Vi" was used to designate the new antibody. The corresponding antigen was termed Virulence Antigen, or "Vi Antigen".

Further experiments by Felix and Pitt (1935), Felix, Krikorian and Reitler (1935), and Felix et al (1935), provided for better understanding of the Vi antigen. Independence of the Vi antibody was established by absorption tests. This was confirmed by Dyachenko (1936). The Vi antibody was more protective than O antibody, and the combination of Vi and O antibody neither increased nor diminished that protective capacity. An explanation for this protective ability was offered by Bhatnagar (1935) who reported that inagglutinable strains were highly resistant to phagocytosis, while agglutinable strains were highly susceptible.

The controversy developed as to whether Vi antigen was actually representative of virulence. Felix (1936) maintained that it was, despite the fact that Vi antigen was present in avirulent rough variants of virulent smooth strains. Kauffmann and Orskov (1936) related virulence to toxic

conditions in mice. Large doses of living Vi forms were reported to cause acute intoxication followed by death in two days. With smaller, non-intoxicating doses it was not possible to demonstrate any difference between Vi and non-Vi forms of the same strain, with respect to severity of infection. They concluded that Vi antigen plays no part in virulence of typhoid bacilli for mice. Almon (1943) attributes the toxic effect of Vi antigen in large doses to superior multiplication of the Vi organism. The situation of Vi substance near the surface of the antigen has also been suggested as a possible key to the genesis of virulence.

It was noted that some Vi properties could be altered simply by routine transfer of a culture. Changes were determined by agglutination and absorption tests. The transformation from Vi to non-Vi was gradual and involved an intermediate condition in which the original resistance to O agglutination was lost but agglutination in Vi anti-serum was retained. Kauffmann (1935) introduced a terminology pertaining to these changes. Those organisms containing Vi antigen and demonstrating O inagglutinability were termed "V" forms (viel). Intermediate conditions were termed "V-W" forms (transition). Finally, when all Vi characteristics were lost the organisms were called "W" forms (wenig). Kauffmann believed that V-W transition was reversible but this condition is known to be rare.

Felix and Pitt (1934a) demonstrated the presence of

Vi antigen by agglutinin absorption tests, as previously mentioned. Serological selection of this type entails considerable time and labour. Kauffmann's (Craigie and Brandon, 1936) slide agglutination method has proved less complex and more accurate.

It was not until Craigie and Brandon (1936) discovered a Vi bacteriophage, that a more rapid and reliable method for detecting Vi antigen was introduced. It was noted by these workers that the Vi form of S. typhosa was sensitive to a bacteriophage isolated from the stool of infected persons. Sensitivity was manifested by lytic areas on the surface of inoculated plates. W forms remained invulnerable to bacteriophage activity. Mixed cultures of V-W forms were detected by their presentation of typical "moth-eaten" areas where inhibition of Vi and non-inhibition of W forms were simultaneously present.

By examining the various strains of bacteriophage active against S. typhosa, Craigie and Yen (1938) were able to isolate a group of four types of Vi bacteriophages (Table I). It was observed in particular that 'Phage II propagation on a strain of S. typhosa resulted in the development of a highly selective, lytic activity for the substrate strain. The advantages of this response were utilized toward determining the identity of Vi strains in unknown cultures.

It was assumed by Craigie and Yen (1938), that varieties of S. typhosa occurred which differed only in their

TABLE I

## Properties of the Four Vi Bacteriophages

| Vi<br>bacterio-<br>phage | Relative<br>particle<br>size | Thermal<br>death<br>point | Neutralization<br>by antiphage<br>serum |    |     |    | Lytic activity<br>for Vi forms of<br><u>S. typhi</u>                                                              |
|--------------------------|------------------------------|---------------------------|-----------------------------------------|----|-----|----|-------------------------------------------------------------------------------------------------------------------|
|                          |                              |                           | I                                       | II | III | IV |                                                                                                                   |
| Type I                   | Large<br>'phage              | 67-70°C                   | -                                       | -  | -   | -  | Lyses all Vi<br>forms                                                                                             |
| Type II                  | Medium<br>'phage             | 69-72°C                   | -                                       | -  | -   | -  | Develops a high<br>selective lytic<br>activity for the<br>type of <u>S. typhi</u><br>on which it is<br>propagated |
| Type III                 | Small<br>'phage              | 61-64°C                   | -                                       | -  | -   | -  | Lyses majority<br>of Vi forms                                                                                     |
| Type IV                  | Medium<br>'phage             | 59-62°C                   | -                                       | -  | -   | -  | Lyses majority<br>of Vi forms with<br>exceptions of<br>Types F and D <sub>1</sub>                                 |

From the above information, Craigie and Yen (1938)  
deduced several facts:

1. The four bacteriophages are specific for the Vi form of S. typhosa.
2. Serologically, the four types of bacteriophage are distinct.
3. A variation occurs in the lytic action on Vi form strains, particularly in the case of Type II bacteriophage.



Vi antigen components. Consequently, they postulated that 'Phage II exists as a series of mutants differing from each other in selective affinity for different Vi strains of S. typhosa. The loss of unrelated mutant particles with concurrent development of a specific mutant on the substrate strain, probably initiated the increase in specificity of bacteriophage.

By continuous selective propagation of these mutants on a substrate strain, it was possible to obtain bacteriophage preparations which manifested a highly developed affinity for the homologous Vi strain of S. typhosa.

Applying this method of selective propagation, Craigie and Yen (1938) produced the following eleven bacteriophages:

A, B<sub>1</sub>, B<sub>2</sub>, C, D<sub>1</sub>, D<sub>2</sub>, E<sub>1</sub>, F<sub>1</sub>, G, H and L. At present, the complete list of known types and subtypes includes A, B<sub>1</sub>, B<sub>2</sub>, B<sub>3</sub>, C, D<sub>1</sub>, D<sub>2</sub>, D<sub>4</sub>HP<sub>56</sub>, D<sub>5</sub>, D<sub>6</sub>, E<sub>1</sub>, E<sub>2</sub>, F<sub>1</sub>, F<sub>2</sub>, G, H, J, K, L<sub>1</sub>, L<sub>2</sub>, M, N, O and T. The reactions of these bacteriophages against their corresponding Vi type strains are tabulated in Table II.

The specificity of Vi type strains may be questioned since Type A displays sensitivity to all Vi bacteriophage preparations. However, this is of rare occurrence. Craigie and Felix (1947) recently indicated that this may be due to dissociation from the original Vi type or types present in a carrier.

The application of bacteriophage typing in diagnostic laboratories has established its value for accurate deter-





mination of the presence of Vi antigen as well as the specific type present.

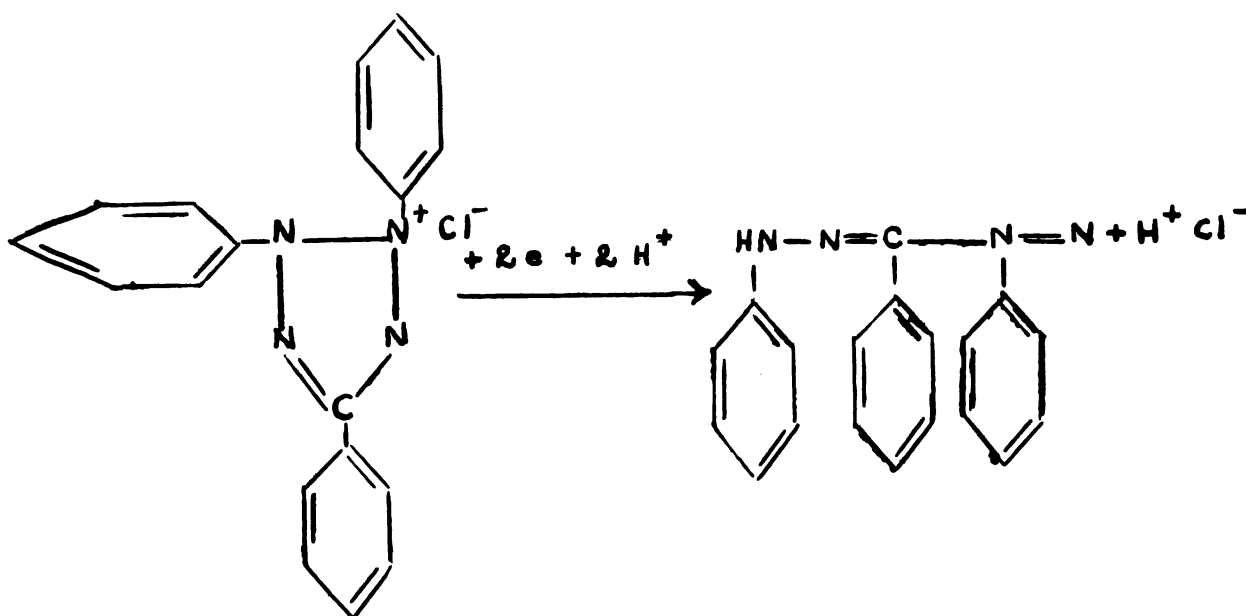
The differentiation between Vi and W forms by visual means has been more recently described by Landy (1950). With the optical arrangement properly adjusted, Vi forms appear very dense and reddish-coppery in color, while the W forms are translucent and appear grayish green. The degree of density of the colony and the coppery appearance is associated with the quantity of Vi antigen present. It has been previously stated that this procedure demands the efforts of those who are highly skilled and therefore competent in detecting the subtle points for differentiation.

TETRAZOLIUM

In recent years a new compound, 2,3,5-triphenyl tetrazolium chloride, has gained the interest of bacteriologists as a prospective indicator of colonial differences in bacterial variants. For the purpose of simplicity the salt will hereafter be referred to as tetrazolium.

The advantages of tetrazolium were originally discovered in Germany by a botanist (Lakon, 1942). He applied the salt as a means of determining the viability of seeds. Its practical value was recognized in this country and soon adopted for purposes of a similar nature.

Mattson et al (1947) revealed that living tissues will reduce the colorless tetrazolium to form an insoluble, triphenyl formazan which is red. They reported that the reduction is produced by the following reaction:



The reaction was described as a result of enzyme activity present in living tissue. It was indicated more specifically, that this activity was probably a dehydrogenase system requiring coenzymes I and II as the electron acceptor. Thus, variations occurring in the enzyme activity of an organism, will reduce the salt accordingly.

Lederberg (1948) found that tetrazolium was instrumental in determining the presence of fermenters and non-fermenting variants of Escherichia coli. Non-fermenting variants stained red, whereas the fermenters produced no change.

Levine and Garber (1950) speculated on the effects of tetrazolium on dissociants of Pasturella pestis. They found that smooth and rough colonies could be readily identified when cultivated on tryptose agar containing tetrazolium. Smooth colonies were approximately 2 mm. in diameter, round, and exhibited sharply defined carmine red centers. Rough colonies were irregular in outline and presented by a diffused pink color.

That same year, Huddleson (1950) published a more conclusive report of results obtained in subjecting various bacterial species to tetrazolium. He observed differences in color and opacity of the borders surrounding the colonies as well as varying degrees of color intensity within the borders. He particularly described the effects produced on the species of Brucella. The S phase colonies of Brucella suis stained dark red in the central portion, while the borders were light opaque yellow. In

contrast, Brucella abortus stained lighter red in the central portions with light, almost transparent, bluish green borders. Finally, Brucella melitensis stained the lightest red in the central portions with light opaque pink borders. Huddleson's work was not restricted to the variations occurring in the Brucella species, but included various phases occurring within the species of Salmonella, Shigella, Escherichia, Alcaligenes, Streptococcus and some aerobic spore-forming bacilli.

In view of these results, tetrazolium was employed as a possible indicator in the differentiation between the Vi and W forms of S. typhosa.

## MATERIALS

The four Vi-type cultures of S. typhosa: A, D<sub>4</sub>HP<sub>56</sub>, E<sub>1</sub>, and F<sub>1</sub>, and their homologous Vi bacteriophage strains in concentrated form, originated from the Connaught Medical Research Laboratories, University of Toronto, Toronto, Canada. They were subsequently received by this laboratory through the Michigan State Department of Health, Lansing, Michigan. Type H901 was received upon request from Dr. W. Ferguson of the Michigan State Department of Health.

All the Vi-type cultures were maintained on Dorset Egg medium (3 parts whole egg, 1 part saline, slanted and inspissated). Stock strains were transferred to new slants at five month intervals following passage on nutrient agar and tests for specificity. 'Bacto' Tryptose Agar slants were used in making daily transfers.

A preliminary test on each concentrated Vi-'phage type was determined to obtain its Critical Test Concentration (C.T.C.) which is the greatest dilution of bacteriophage that will produce inhibition on the homologous strain of S. typhosa. This resulted in diluting each bacteriophage to 1:100.

The following media were prepared for bacteriophage typing, as recommended by Craigie and Yen (1938):



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## Agar medium

|                                     |             |
|-------------------------------------|-------------|
| 'Bacto' nutrient broth (dehydrated) | 20.00 gms.  |
| Sodium chloride                     | 7.50 gms.   |
| 'Bacto' agar                        | 20.00 gms.  |
| Distilled water to                  | 1000.00 ml. |

## 'Phage dilution broth

|                                     |             |
|-------------------------------------|-------------|
| 'Bacto' nutrient broth (dehydrated) | 8.00 gms.   |
| Distilled water to                  | 1000.00 ml. |

## 'Phage culture broth

|                                     |             |
|-------------------------------------|-------------|
| 'Bacto' nutrient broth (dehydrated) | 15.00 gms.  |
| Sodium chloride                     | 7.00 gms.   |
| Distilled water to                  | 1000.00 ml. |

The tetrazolium, in powder form, was obtained from the Montclair Research Corporation, Montclair, New Jersey. This material was stored in a dark place as accompanying instruction specified. A 1 percent aqueous solution was prepared and sterilized at 115° C for 15 minutes. This was added to 'Bacto' tryptose agar in the following manner: 1 ml. of 1 percent aqueous solution of tetrazolium was transferred to 100 ml. of sterile, melted tryptose agar. The agar was cooled before adding the tetrazolium solution. This combination was mixed well to insure uniform distribution. The mixture was then poured in Petri dishes approximately 0.5 cm. in depth, and incubated at 37° C for about twenty hours to remove excess moisture from their surfaces.

Tube 1 McFarland's nephelometer was used as a turbidi-

metric standard for estimating the dilution of a culture suspension. The density of Tube 1 McFarland's nephelometer represents a bacterial suspension approximating three hundred million organisms (Todd and Sanford, 1948).

Dilution blanks containing 99 ml. of sterile physiological saline solution was used to reduce the concentration of organisms.

## METHODS

All cultures were typed to determine the presence, or absence of Vi antigen, concurrently with inoculation on tetrazolium-agar plates. Typing procedures were duplicated as recommended by Craigie and Yen (1938) in every detail.

A small amount of the growth on tryptose agar slants was inoculated into 2 ml. of nutrient broth and allowed to incubate at 37° C for 2-4 hours. At the end of this period, nutrient agar plates were marked for areas which were inoculated in the following manner. A loopholder with a standard platinum loop (2.75 mm. in internal diameter), was supported between the thumb and first two fingers like a pen. A full loopful of the broth culture was obtained by inserting the loop horizontally without touching the sides of the tube and quickly removing it while in the horizontal position. With the forearm and side of the hand resting on the bench, the drop was transferred to the marked area on the agar plate by merely touching the surface of the agar. The loop was then horizontally moved about an imaginary circle by manipulating the fingers over an area approximately 15 mm. in diameter. Thus, the drop is allowed to spread in a natural way. The loop was actually used as a guide and never allowed to come in contact with the surface of the agar. This caution is to be observed

to prevent pseudo-lytic areas.

When the fluid had become absorbed, by the agar, (5-10 minutes), the bacteriophage was applied to the center of the inoculated area. In this case, the loopholder was supported between the thumb and fingers in a horizontal position. The loopful of bacteriophage was lowered until it almost touched the surface of the agar, at which time, it was rotated into a vertical position allowing the bacteriophage to flow onto the center of the inoculated area. Once again, actual contact of the loop and agar was avoided.

When the plates were thoroughly dried, they were incubated for 2 hours at 37° C. They were then removed to the refrigerator overnight followed by reincubation for 4-6 hours. The reactions were observed for lytic areas against a dark background.

The method of interrupted incubation is preferable in view of the following advantages. Bacteriophage reactions may be observed at their optimum activity before they are obscured by overgrowth by some strains. The interval of storage in the cold room permits diffusion of the bacteriophage into the surrounding normal culture.

As previously stated, cultures were typed concurrently with inoculation of tetrazolium-agar plates. The procedure for inoculation was as follows: a small amount of growth was removed from a tryptose agar slant and inoculated into

a tube containing 10 ml. of sterile, physiological saline solution. The organisms were dispersed in the solution with the aid of the loop and if necessary, by agitating the tube. This was compared with the Tube 1 nephelometer. If the suspension was too light or too heavy, it was corrected by a heavier inoculation of organisms or dilution with sterile saline as required. With the aid of a graduated pipette, 0.1 ml. of the culture suspension was transferred to a dilution blank containing 99 ml. of sterile, physiological saline solution. After mixing well, 0.1 ml. was removed and transferred into a second dilution blank, which was also mixed well. Finally, 0.5 ml. was removed from the second blank and transferred onto the tetrazolium-agar plate. This was rotated to insure uniform expansion of the suspension. The plate was allowed to dry at room temperature on a table or board with a level surface.

When the suspension had been absorbed, the plates were inverted and incubated at 37° C for 48 hours. The resultant red colonies were examined for various color patterns as recommended by Huddleson (1950). A plate was placed "under a low power (x12) dissecting microscope which was illuminated by reflected, oblique light from the new Spencer microscope lamp, Model #735. The light was focused at an angle of 40° on a small, concave mirror placed in front of the microscope. The rays were reflected at an oblique angle from the mirror so as to strike the under surface of the Petri plate resting on the glass stage."

To stimulate the transformation from the Vi form to the W form, several series of cultures corresponding to the stock strains were transferred daily, to fresh tryptose agar slants.

TABLE III

## Color Patterns of Type A Strain

| Time     | Vi 'phage<br>typing | Colonies | Color patterns |                  |
|----------|---------------------|----------|----------------|------------------|
|          |                     |          | Borders        | Central portions |
| 4 weeks  | CL                  | S        | N, LY0         | IDCR             |
| 8 weeks  | CL                  | S        | N, LY0         | IDCR             |
| 12 weeks | CL                  | S        | N, LY0         | UR               |
| 16 weeks | CL                  | S        | N, LY0         | UR               |

CL - Confluent lysis    LY0 - Light Yellow Opaque  
 S - Smooth            IDCR - Interior Deep Crimson Red  
 N - Narrow            UR - Uniform Red

TABLE IV

Color Patterns of Type D<sub>4</sub>HP<sub>56</sub>

| Time     | Vi 'phage<br>typing | Colonies | Color patterns |                  |
|----------|---------------------|----------|----------------|------------------|
|          |                     |          | Borders        | Central portions |
| 4 weeks  | CL                  | S        | N, LY0         | IDCR             |
| 8 weeks  | -                   | S        | N, Bl 0        | UR               |
| 12 weeks | -                   | Ro       | W, BT          | R                |
| 16 weeks | -                   | Ro       | W, BT          | R                |

CL - Confluent lysis    LY0 - Light Yellow Opaque  
 - - No lysis            IDCR - Interior Deep Crimson Red  
 S - Smooth            UR - Uniform Red  
 N - Narrow            BT - Blue, transparent  
 W - Wide              R - Radial  
 Bl 0 - Bluish Opaque    Ro - Rough



TABLE V

Color Patterns of Type E<sub>1</sub> Strain

| Time     | Vi 'phage typing | Colonies | Color patterns |                  |
|----------|------------------|----------|----------------|------------------|
|          |                  |          | Borders        | Central portions |
| 4 weeks  | CL               | S        | N, LYO         | IDCR             |
| 8 weeks  | ⌢                | S        | N, LYO         | UR and R         |
| 12 weeks | -                | S        | N, Bl O        | UR and R         |
| 16 weeks | -                | S        | W, BT          | UR and R         |

CL - Confluent lysis    LYO - Light Yellow Opaque  
 - - No lysis            IDCR - Interior Deep Crimson Red  
 ⌢ - Partial lysis      UR - Uniform Red  
 N - Narrow              Bl O - Bluish Opaque  
 W - Wide                BT - Blue Transparent  
 S - Smooth              R - Radial

TABLE VI

Color Patterns of Type F<sub>1</sub> Strain

| Time     | Vi 'phage typing | Colonies | Color patterns |                  |
|----------|------------------|----------|----------------|------------------|
|          |                  |          | Borders        | Central portions |
| 4 weeks  | CL               | S        | N, LYO         | IDCR             |
| 8 weeks  | ⌢                | S        | N, LYO         | UR and R         |
| 12 weeks | -                | Ro       | W, BT          | UR and R         |
| 16 weeks | O                | O        | O              | O                |

CL - Confluent lysis    N - Narrow  
 - - No lysis            W - Wide  
 ⌢ - Partial lysis      LYO - Light Yellow Opaque  
 O - No growth        IDCR - Inter Deep Crimson Red  
 S - Smooth            UR - Uniform Red  
 Ro - Rough            BT - Blue Transparent  
                          R - Radial

TABLE VII

Color Patterns of Type F<sub>1</sub> Strain\*

| Time     | Vi 'phage<br>typing | Colonies | Color patterns |                  |
|----------|---------------------|----------|----------------|------------------|
|          |                     |          | Borders        | Central portions |
| 4 weeks  | CL                  | S        | N, LYO         | IDCR             |
| 8 weeks  | CL                  | S and Ro | N, LYO         | IDCR and R       |
| 12 weeks | CL                  | S and Ro | N, LYO         | IDCR and R       |

CL - Confluent lysis    LYO - Light Yellow Opaque  
 S - Smooth              IDCR - Interior Deep Crimson Red  
 Ro - Rough               R - Radial  
 N - Narrow

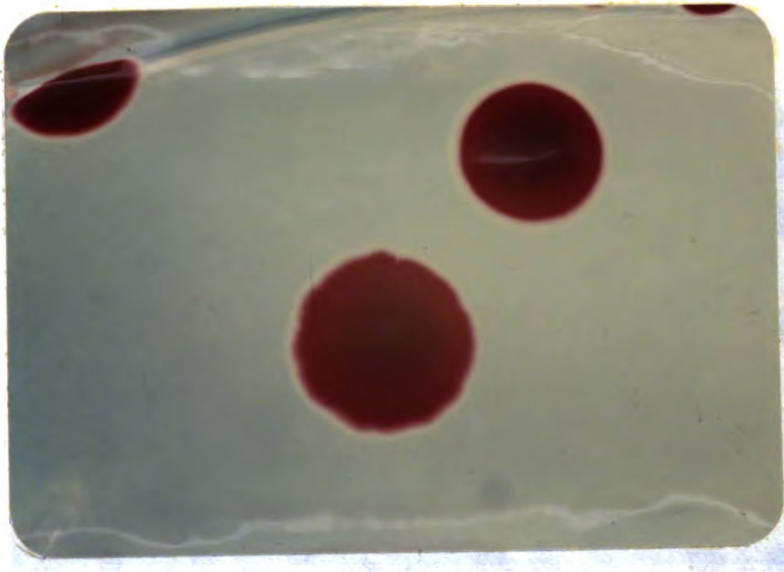
\* This strain was requested from the Michigan State Department of Health when its preceding corresponding strain had become non-viable.

TABLE VIII

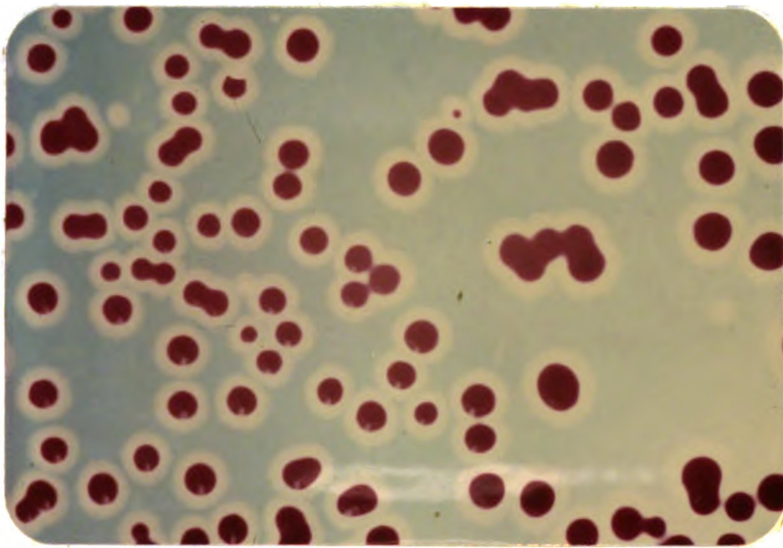
## Color Patterns of Type H 901 Strain

| Time         | Vi 'phage<br>typing | Colonies | Color patterns |                  |
|--------------|---------------------|----------|----------------|------------------|
|              |                     |          | Borders        | Central portions |
| Upon receipt | -                   | S and Ro | W, BT          | UR               |

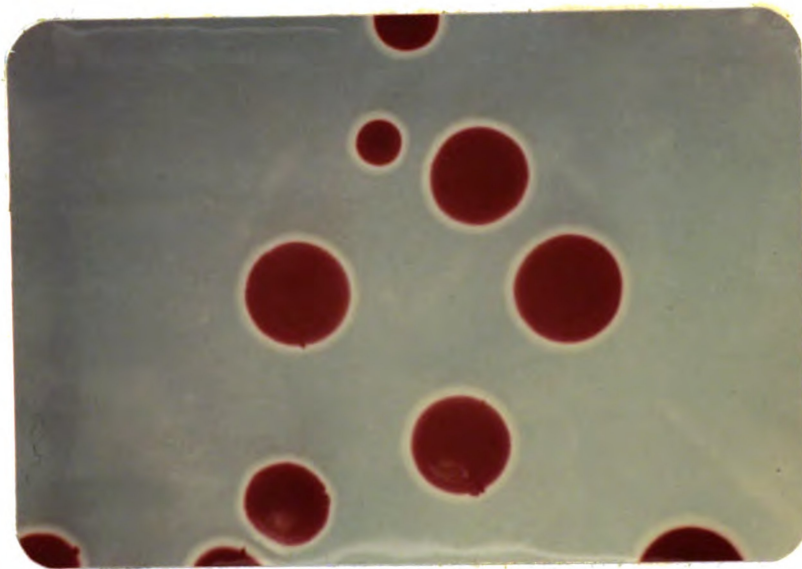
- - No lysis              W - Wide  
 S - Smooth               BT - Blue Transparent  
 Ro - Rough               UR - Uniform Red



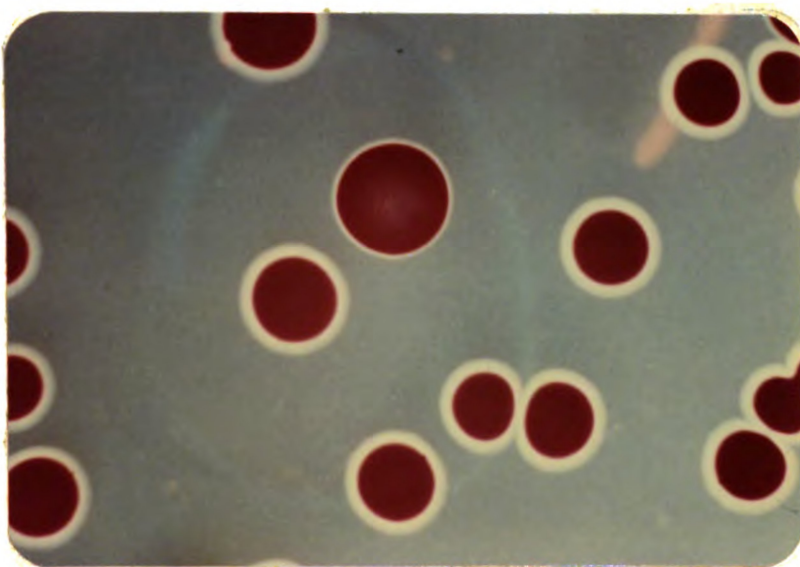
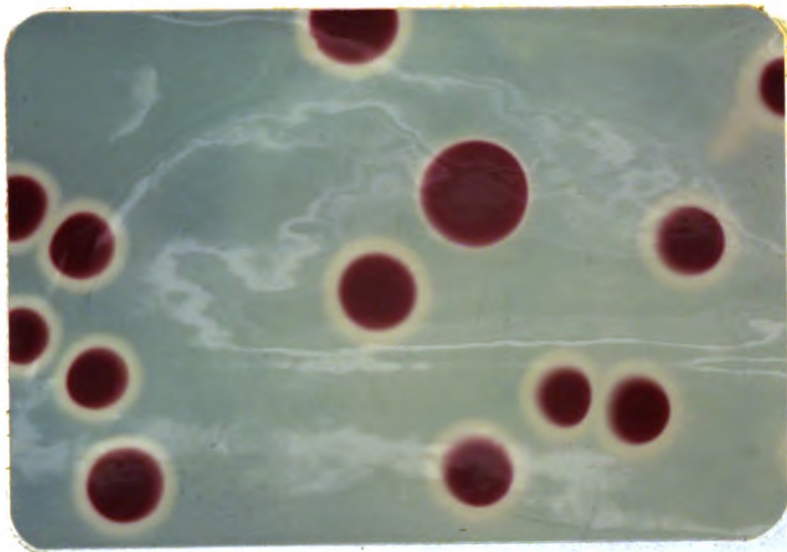
Smooth and Rough Vi Antigen Colonies



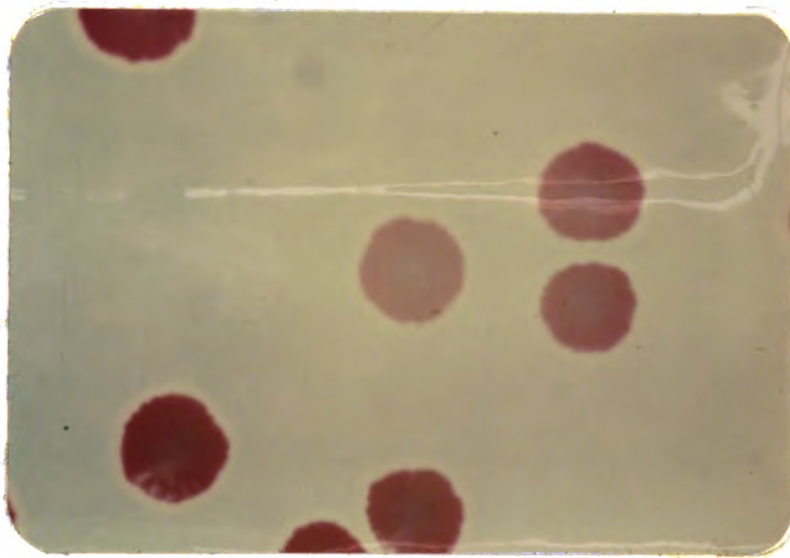
W Colonies



V-W Colonies



Single Vi Antigen Colony Among W Colonies  
(Taken with two different time exposures)



W Colonies with Radial Pattern

## DISCUSSION

It may be seen from the preceding tables that various color patterns are demonstrated during increased periods of subcultivation. At the beginning of the study, when types A, D<sub>4</sub>HP<sub>56</sub>, E<sub>1</sub>, and F<sub>1</sub> exhibited confluent lysis, comparatively identical color patterns were expressed. Collectively, the significant features were presented by light yellow opaque borders accompanied by an innermost deep, crimson red center. The edges of these centers were sharply defined and were surrounded by an area of lighter colored red. This indicates the presence of Vi antigen.

As the study progressed and Vi antigen gradually was destroyed, the well defined central areas became successively less defined until they displayed a uniform light red color throughout to the borders. At the same time, the borders became less opaque assuming translucency. The light yellow of the opaque borders finally became bluish when completely translucent. The bluish, translucent patterns indicate strains which are devoid of Vi antigen, the W form.

Of the individual strains, Type A particularly persisted in retaining the Vi characteristics. At no time did the borders lose their opacity, although central areas became light red throughout to the borders after 8 weeks of continuous subcultivation. The gradual recession of the deep crimson centers were observed as the initial manifestation of Vi antigen deterioration.



Types  $E_1$  and  $F_1$  produced a similar pattern of results during the course of the study. Both strains completely lost their Vi characteristics after a period of 12 weeks. This was represented by a progressively wider border, becoming more translucent with age after their centers lost the significant deep, crimson red. The varying degrees of these patterns are indicative of the transitional, V-W forms.

Type  $D_4HP_{56}$  became devoid of the Vi antigen after only 4 weeks of subcultivation. This too, was demonstrated by wide, bluish, transparent borders with light red centers. The colonies of this strain assumed roughness comparatively early until their irregularity could be easily detected without the aid of a microscope. The early loss of Vi antigen of Type  $D_4HP_{56}$  may be due to its variability in type. It has been suggested (Henderson and Ferguson, 1949) that Type  $D_4HP_{56}$  cultures, upon storage, tend to change their reactions with Type II 'Phage to Type  $D_1$ . The possibility that Type  $D_4HP_{56}$  carries a latent bacteriophage which produces comparable reactions when tested on Vi type stock strains and W form cultures of S. typhi, may account for the comparatively early demonstration of the W form.

The non-significance of rough and smooth colonies in relation to Vi antigen is clearly presented. Both types of colonies showed Vi, W and V-W forms without distinguishing.

Type H 901 was received at the close of the study.

This is a fixed, classic W form which has been used as a standard strain in research laboratories for many years. The color patterns of this strain were therefore compared with those W forms which were induced by subcultivation. Essentially the same patterns were presented. The only shades of difference detected were found in slightly wider borders with more translucency among colonies of Type H 901.

It is noted that a number of plates produced a radial pattern in the central portions. There was no apparent reason for which this could be attributed. Both Vi and W forms demonstrated this pattern. It was suspected that these patterns were characteristic of the transitional, V-W forms, since they were first noticed in cultures which were partially lysed when typed with homologous bacteriophage strains. However, variations of the basic pattern occurred in pure Vi and W forms. Further, when plates were re-examined after storage in the refrigerator for periods of 2, 3, and 4 months, it was noted that the radial patterns became more pronounced. They also commenced to make their appearance in colonies which did not demonstrate them originally.

It is possible that a reversion to the colorless characteristic of tetrazolium was in process. The reaction producing color is due to an oxidation-reduction system involving enzymatic activity. Perhaps these organisms which were gradually losing the activity of their enzyme

systems, or, as in the other cases, which might be weak in the activity of their enzyme systems resulted in the salt resuming its oxidized form. If a growth estimate could be calculated to determine the decrease in viable organisms, or the degree of activity present, some correlation might be presented to explain these effects.

It is also possible that the deep, crimson red centers present in colonies of the Vi form were the result of superior activity of their enzyme systems. The Vi forms have been suggested to be more active in growth and reproduction, a factor contributing to their virulence. A colony of organisms multiplying more rapidly would elicit more activity of their enzyme systems.

The object in inoculating the plates with a suspension was mainly to obtain a limited number of well isolated colonies. Also, if plates were streaked rather than poured with suspensions, a slightly but evident growing out from the sides of the colonies occurred simulating the pattern made by the streak lines. This would tend to obscure the true pattern of the colonies.

The plates required 48 hours incubation for complete and accurate color development. Upon observation of colonies at earlier periods of incubation, it was noticed that all forms presented colonies which were indistinguishable from one another.

## SUMMARY

A study is presented to demonstrate the use of 2,3,5-triphenyl tetrazolium chloride as an indicator, which when incorporated in a medium, will manifest a visible distinction between the colonies of typhoid Vi and W antigens.

The results show that those strains possessing completeness of the Vi antigen, conform to a significant pattern. This pattern may be described as demonstrating a sharply defined, deep, crimson red center, surrounded by an area of lighter red. A border encircling the colony is narrow, and in all cases opaque, with slight color variations ranging from light yellow to light rose.

The W forms, completely devoid of the Vi antigen, present patterns which are readily distinguished from those of the Vi forms. The sharply defined, deep, crimson red center is completely absent. Rather, the central portion is a uniform light red. A border encircling the colony is about two to three times wider than that of the Vi forms, and in all cases translucent, with slight color variations ranging from light blue to light green.

The V-W forms are demonstrated by a combination of patterns associated with those of the individual forms in their pure state. That is, a section of the colony may be typically representative of a W form, while the remainder of the colony is clearly characteristic of a Vi form.

There were a considerable number of colonies which presented a radial pattern. An account of the possible circumstances conducive to such a pattern is presented in the discussion of this paper.

Types A, D<sub>4</sub>HP<sub>56</sub>, E<sub>1</sub>, and F<sub>1</sub> were used in this study. They were all received in the Vi form. By continuous subcultivation, it was possible to induce degradation to the W form. Type A exclusively persisted in its Vi form.

Bacteriophage typing procedure was applied to determine the presence or absence of Vi antigen. A method for the preparation of tetrazolium salt in agar is described.

## BIBLIOGRAPHY

1. Almon, Lois. The significance of the Vi antigen. *Bact. Rev.*, 6-7 (1942-43): 43-53.
2. Almon, L. and Stovall, W. D. A study of Vi antigen of Felix and Pitt. *J. Immunol.*, 31, (1936): 269-284.
3. Andrewes, F. W. Studies in group agglutination. I. Salmonella group and its antigenic structure. *J. Path. and Bact.*, 25 (1922): 505.
4. Arkwright, J. N. Variation in bacteria in relation to agglutination both by salts and by specific serum. *J. Path. and Bact.*, 24 (1921): 36.
5. Atkinson, E., Melvin, S. and Fox, S. Some properties of 2,3,5-triphenyl tetrazolium chloride and several iodo derivatives. *Science*, 111 (1950): 385.
6. Bhatnagar, S. S. Phagocytosis of *B. typhosus* in relation to antigenic structure. *Brit. J. of Exp. Path.*, 16 (1935): 375.
7. Burnet, F. M. The relationships between heat-stable agglutinogens and sensitivity of bacteriophage in the Salmonella group. *Brit. J. of Exp. Path.*, 8 (1927): 121-129.
8. Craigie, James. Typing of typhoid bacilli with Type II phage. *The Cyclopedia of Med., Surg. and Specialties.*, Service Volume, 5 (1941).
9. Craigie, J. and Brandon, K. F. Identification of the V form of *B. typhosus*. *Can. Pub. Health J.*, 27 (1936): 165-170.
10. Craigie, J. and Felix, A. Typing of typhoid bacilli with Vi bacteriophage. *Lancet* (1947): 823-836.
11. Craigie, J. and Yen, Chun Hui. The demonstration of types of *B. typhosus* by means of preparations of type II Vi phage. *Can. Pub. Health J.*, 29 (1938): 448.
12. de Kruif, Paul J. Dissociation of microbic species. *J. of Exp. Med.*, 33 (1921): 773.
13. Detre, V. Purification of Vi type *B. typhosus* strains. *J. of Inf. Dis.*, 60 (1937): 319.
14. Dyachenko, S. S. A contribution to the problem of the virulence (Vi) antigen of *B. typhosum* Ty 2. *J. Hyg.*, 36 (1936): 108-113.

15. Edwards, P. R. and Bruner, D. W. Serological identification of Salmonella cultures. Agricultural Exp. Station, Univ. of Ky., Circular 54 (1942).
16. Felix, A. P. Experience with typing of typhoid bacilli by means of Vi bacteriophage. Brit. Med. J., 1 (1943): 435-438.
17. Felix, A. P., Bhatnagar, S. S. and Pitt, R. M. Further observations on the properties of the Vi antigen of E. typhosus and its corresponding antibody. J. Exp. Path., 16 (1935): 422.
18. Felix, A. P., Krikorian, K. S. and Reitler, R. The occurrence of typhoid bacilli containing Vi antigen in typhoid fever. J. Hyg., 35 (1935): 421.
19. Felix, A. P. and Pitt, R. M. A new antigen of Bacterium typhosus: its relation to virulence and to active and passive immunization. Lancet, 2 (1934a): 186.
20. Felix, A. P. and Pitt, R. M. Virulence of E. typhosus and resistance to O antibody. J. of Path. and Bact., 38 (1934): 409.
21. Felix, A. P. and Pitt, R. M. Virulence and immunogenic activities of E. typhosus in relation to its antigenic constituents. J. Hyg., 35 (1935): 428.
22. Felsenfeld, O. The Salmonella problem: practical laboratory applications of recent advances. Amer. J. of Clin. Path., 15 (1945): 584.
23. Grinnell, F. B. A study of the comparative value of rough and smooth strains of E. typhosus on the preparation of typhoid vaccines. J. of Immun., 19 (1930): 457.
24. Henderson, N. D. and Ferguson, W. W. Bacteriophage typing of S. typhosa. J. of Lab. and Clin. Med., 34 (1949): 739-743.
25. Henderson, N. D. and Ferguson, W. W. Investigation of bacteriophage types D<sub>1</sub> and D<sub>4</sub><sup>HP</sup><sub>56</sub> of S. typhi and their latent bacteriophages. Amer. J. of Pub. Hyg., 50 (1949): 349-359.
26. Huddleson, I. F. and Baltzer, B. Differentiation of bacterial species and variation within species by means of 2,3,5-triphenyl tetrazolium chloride in culture medium. Science, 112 (1950): 651.
27. Kauffmann, F. Über einen neuen serologischen Formenwechsel der Typhus-bacillen. Zeit. für Bakt. und Infect., 116 (1934-35): 617-651.

28. Kauffmann, F. A typhoid variant and a new serological variation in the Salmonella group. J. Bact., 41 (1941): 127.
29. Kauffmann, F. and Moller, H. A new type of Salmonella (S. ballerup) with Vi antigen. J. Hyg., 37 (1940): 246.
30. Kauffmann, F. and Orskov, J. Studies on the significance of Vi antigen in the mechanism of typhoid fever infection in mice. J. Hyg., 36 (1936): 514.
31. Klein, M. The Vi antigen in the detection of typhoid carriers. J. of Inf. Dis., 72 (1943): 49-57.
32. Lakon, G. The topographical tetrazolium method for determining the germinating capacity of seeds. Plant Physiol., 24 (1949): 389-394.
33. Landy, M. The visual identification of V and W form colonies in Salmonella cultures. Public Health Reports, July 28, 1950: 950.
34. Lederberg, J. Detection of fermentative variants with tetrazolium. J. Bact., 56 (1948): 695.
35. Levine, H. B. and Garber, E. D. Detection of rough dissociants of Pasturella pestis with tetrazolium chloride. J. Bact., 60 (1950): 508.
36. Mattson, A. M., Jensen, C. O. and Dutcher, R. A. Triphenyl tetrazolium chloride as a dye for vital tissues. Science, 106 (1947): 294.
37. Peluso, C. A. Stability of the Vi antigen of S. typhosa. Proc. Soc. Exp. Biol. and Med., 48 (1941): 340.
38. Penfold, W. J. Studies in bacterial variation. J. Hyg., 11 (1910): 30.
39. Robertson, R. C. and Yu, H. The Vi antigen of B. typhosus. J. of Path. and Bact., 43 (1936): 191.
40. Savage, W. G. Pseudo-clumping in cultures of the typhoid bacillus. J. of Path. and Bact., 7 (1901): 368.
41. Todd, J. C. and Sanford, A. H. Clinical diagnosis by laboratory methods. 11th edition, (1948): 850.
42. White, P. B. Notes on intestinal bacilli with special reference to smooth and rough races. J. of Path. and Bact., 32 (1929): 85-94.



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